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Short communication

Daily intake of β -cryptoxanthin prevents bone loss by preferential disturbance of osteoclastic activation in ovariectomized miceKakeru Ozaki ^{a,1}, Maika Okamoto ^{a,1}, Kazuya Fukasawa ^a, Takashi Iezaki ^a, Yuki Onishi ^a, Yukio Yoneda ^a, Minoru Sugiura ^b, Eiichi Hinoi ^{a,*}^a Laboratory of Molecular Pharmacology, Division of Pharmaceutical Sciences, Kanazawa University Graduate School of Natural Science and Technology, Kanazawa, Ishikawa, Japan^b Citrus Research Division, NARO Institute of Fruit Tree Science, National Agriculture and Food Research Organization, Shimizu, Shizuoka, Japan

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ABSTRACT

Although β -cryptoxanthin, a xanthophyll carotenoid, has been shown to exert an anabolic effect on bone calcification, little attention has been paid thus far to the precise mechanism of bone remodeling. Daily oral administration of β -cryptoxanthin significantly inhibited osteoclastic activation as well as reduction of bone volume in ovariectomized mice. *In vitro* studies revealed that β -cryptoxanthin inhibited differentiation and maturation of osteoclasts by repression of the nuclear factor- κ B-dependent transcriptional pathway. Our results suggest that supplementation with β -cryptoxanthin would be beneficial for prophylaxis and for therapy of metabolic bone diseases associated with abnormal osteoclast activation.

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Bone homeostasis is complexly regulated by bone-resorbing osteoclasts and bone-forming osteoblasts (1). Osteoclasts are multinucleated cells derived from hematopoietic stem cells, whereas the osteoblast lineage is derived from primitive multipotent mesenchymal stem cells. Imbalance between regulation by osteoclasts and by osteoblasts results in certain metabolic bone diseases including osteoporosis (2).

β -Cryptoxanthin, a major carotenoid routinely found in human serum, is primarily obtained from citrus fruits (3). Several independent lines of evidence suggest a relationship between β -cryptoxanthin and pathophysiology (4). β -cryptoxanthin alleviated diet-induced nonalcoholic steatohepatitis by suppressing inflammatory gene expression (5). In addition, a high serum β -cryptoxanthin level is associated with a lower risk of lung cancer death (6). Moreover, serum β -cryptoxanthin is inversely associated with the risk of human osteoporosis (7), and β -cryptoxanthin shows an anabolic effect on bone calcification in both *in vitro* and *in vivo* studies (8,9). Although several studies have been performed, the

mechanisms of bone remodeling by β -cryptoxanthin remain unclear at the cellular and molecular levels. In the present study, we attempt to show a role and mechanisms of β -cryptoxanthin in the pathogenesis of bone diseases.

The protocol employed here meets the guidelines of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. Eight-week-old female ddY mice were subjected to ovariectomy (OVX) or sham operation in aseptic environments, as described previously (10). Ovariectomized mice received daily oral supplementation of β -cryptoxanthin freshly dissolved in drinking water at concentrations of 1 mg/L or 10 mg/L. Nonesterified β -cryptoxanthin for experiments was prepared and processed as described, and the purity of the β -cryptoxanthin obtained was 96% according to HPLC analysis (5). No significant change was observed in the amount of daily intake of drinking water by mice, irrespective of the concentrations of β -cryptoxanthin used (data not shown). Mice were killed by decapitation 28 days after operation, followed by dissection of vertebrae and subsequent fixation with 10% formalin. Bone histomorphometric analyses were performed on vertebrae, as previously described (11). In brief, the bone volume-to-tissue volume (BV/TV) ratio was measured by Von Kossa staining. Bone formation rate (BFR) was analyzed by calcein double-labeling. Mice were injected twice with calcein at a 3-day interval and were killed

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2 days after the last injection. Osteoblast and osteoclast parameters were analyzed by staining with toluidine blue and with tartrate-resistant acid phosphatase (TRAP), respectively. Analyses were performed with the Osteomeasure Analysis System (Osteometrics, Atlanta, GA), according to standard protocols.

Preosteoclastic RAW264.7 cells were cultured with 20 ng/ml receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL) (R&D systems, Minneapolis, MN, USA) for 4 consecutive days. TRAP staining, actin ring assay, pit formation assay, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay were performed, as previously described (12). For the luciferase assay, cells were transfected with luciferase reporter vectors by the Lipofection method using Lipofectamine LTX/Plus reagent (Invitrogen, Carlsbad, CA, USA) followed by the preparation of cell lysates and determination of luciferase activity using specific substrates in a luminometer (ATTO Corp., Tokyo, Japan). Transfection efficiency was normalized to the activity of *Renilla luc*. Pre-osteoblastic MC3T3-E1 cells were cultured in the presence of differentiation cocktails (50 μ g/ml ascorbic acid and 5 mM β -glycerophosphate). Determination of alkaline phosphatase (ALP) activity and Ca^{2+} accumulation was done as indices of cellular differentiation and maturation, respectively.

All results are expressed as mean \pm standard error. Statistical significance was determined by two-tailed unpaired Student's *t* test or one-way analysis of variance with Bonferroni/Dunnett post hoc test.

We first administered β -cryptoxanthin orally in drinking water to ovariectomized mice for 28 consecutive days at concentrations of 1 mg/L and 10 mg/L. β -Cryptoxanthin administration did not affect body weight of mice, irrespective of OVX (Fig. 1A). Although OVX markedly decreased uterine weight determined 28 days after operation, β -cryptoxanthin did not significantly affect the OVX-induced loss of uterine weight at the concentrations used (Fig. 1B). Under these experimental conditions, a marked reduction was observed in cancellous bone stained by Von Kossa staining in vertebrae of ovariectomized mice compared with that in sham-operated mice (Fig. 1C). However, in vertebrae of ovariectomized mice administered β -cryptoxanthin at 10 mg/L, a marked inhibition of loss of cancellous bone was observed (Fig. 1C). Quantification of these data clearly revealed that supplementation with 10 mg/L β -cryptoxanthin significantly suppressed the reduction of BV/TV ratio in cancellous bone of ovariectomized mice without affecting that in sham-operated mice (Fig. 1D).

To define the cellular basis of the preventive effect of β -cryptoxanthin on bone loss, histomorphometric analyses of vertebrae were performed. In vertebrae of ovariectomized mice, significant increases were observed in the osteoclastic indices, extent of osteoclast surface/bone surface (Oc.S/BS; Fig. 2A and B), and number of osteoclast/bone perimeter (N.Oc/B.Pm; Fig. 2A and C) and in the osteoblastic index, number of osteoblast/bone perimeter (N.Ob/B.Pm; Fig. 2D). The administration of β -cryptoxanthin at 10 mg/L resulted in significant inhibition of the increases of Oc.S/BS and N.Oc/B.Pm to the level observed in sham-operated mice (Fig. 2A–C) but not of elevated N.Ob/B.Pm (Fig. 2D). In addition, the administration of β -cryptoxanthin did not affect BFR, irrespective of OVX (Fig. 2E). Moreover, β -cryptoxanthin at 10 mg/L did not affect BFR of 8-week-old female mice (Fig. 2F). According to the BV/TV ratios, β -cryptoxanthin could affect bone volume by prevention of osteoclastic activation rather than that of osteoblastic function.

We next investigated the possible effect of β -cryptoxanthin on cellular differentiation and maturation of bone-resorbing osteoclasts. For this purpose, preosteoclastic RAW264.7 cells were cultured with RANKL in either the presence or absence of β -cryptoxanthin at concentrations of 1–20 μ M for 4 consecutive days,

followed by TRAP staining for counting TRAP-positive multinucleate cells (MNCs). β -Cryptoxanthin was effective in significantly decreasing the number of TRAP-positive MNCs in a concentration-dependent manner (Fig. 3A and B), but it failed to affect MTT reduction as an index of cellular survival at the concentrations tested (Fig. 3C). Sustained exposure to β -Cryptoxanthin led to the marked decrease in mRNA expression of osteoclastic marker genes, Cathepsin K (*Ctsk*) and *Trap*, in RAW264.7 cells (Fig. 3D). Furthermore, β -cryptoxanthin significantly decreased the number of actin rings (Fig. 3E and F) and area of pit formation (Fig. 3E and G) in RANKL-treated RAW264.7 cells. These results indicate that β -cryptoxanthin prevents the differentiation and maturation of osteoclasts.

Next, we attempted to elucidate the mechanism underlying the inhibition by β -cryptoxanthin of osteoclastic function. Thus, transcriptional activities of NF- κ B, activator protein-1 (AP-1), and the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) (13), all of which are known master transcription factors of osteoclastogenesis, were monitored in RAW264.7 cells. β -Cryptoxanthin significantly repressed the activity of NF- κ B-luc but not that of AP-1-luc or NFATc1-luc (Fig. 3H) despite no marked alteration of mRNA expression in *p65*, *c-Fos*, *c-Jun* and *Nfatc1* (Fig. 3I).

The importance of the present results is that daily oral supplementation of xanthophyll carotenoid β -cryptoxanthin significantly and selectively prevented osteoclastic activation in ovariectomized mice without affecting osteoblastic function, resulting in the inhibition of bone loss *in vivo*. Moreover, β -cryptoxanthin markedly inhibited RANKL-induced osteoclastic differentiation and maturation in association with the inhibition of transcriptional activity of the transcription factor NF- κ B. Although previous studies were indicative of the modulating effects of β -cryptoxanthin on bone formation and bone remodeling *in vivo* (9,14), this is the first demonstration that β -cryptoxanthin prevents bone loss induced by ovariectomy by preferential disturbance of osteoclastic activation rather than by modulation of osteoblastic function.

In this study, we employed 8-week-old ddY female mice for the OVX operation to replicate the *in vivo* experimental conditions often used previously (15). Given that mice are considered to be still undergoing skeletal development at this age, this development could be one reason why β -cryptoxanthin did not significantly prevent the increase in osteoblast numbers with significant amelioration of the increased histomorphometric osteoclast parameters in ovariectomized mice. Indeed, as shown in Fig. 2F, β -cryptoxanthin did not affect BFR of 8-week-old female mice. Moreover, in our preliminary experiments, β -cryptoxanthin, at concentrations affecting osteoclastogenesis (1–20 μ M), did not affect alkaline phosphatase activity or calcium accumulation in osteoblasts *in vitro* (Supplemental Fig. 1A and B), suggesting that osteoblastogenesis cannot be directly regulated by β -cryptoxanthin *in vitro* and *in vivo*. The reason of paradoxical results with previous reports in terms of the anabolic function of β -cryptoxanthin could be at least in part accounted for by taking into consideration of the usage of different animals and cells, developmental stages of animals, or the purity and concentration of β -cryptoxanthin. At any rate, we speculate that β -cryptoxanthin ameliorates the increased osteoclastic parameters through a mechanism relevant to predominant interference with the RANKL/NF- κ B signaling pathway in osteoclasts, without affecting the increased osteoblastic indices, after estrogen deficiency in ovariectomized mice.

The reason why β -cryptoxanthin failed to significantly affect the bone mass in sham-operated mice *in vivo* but inhibited *in vitro* osteoclastogenesis is unclear. It may be that the *in vitro* analysis was

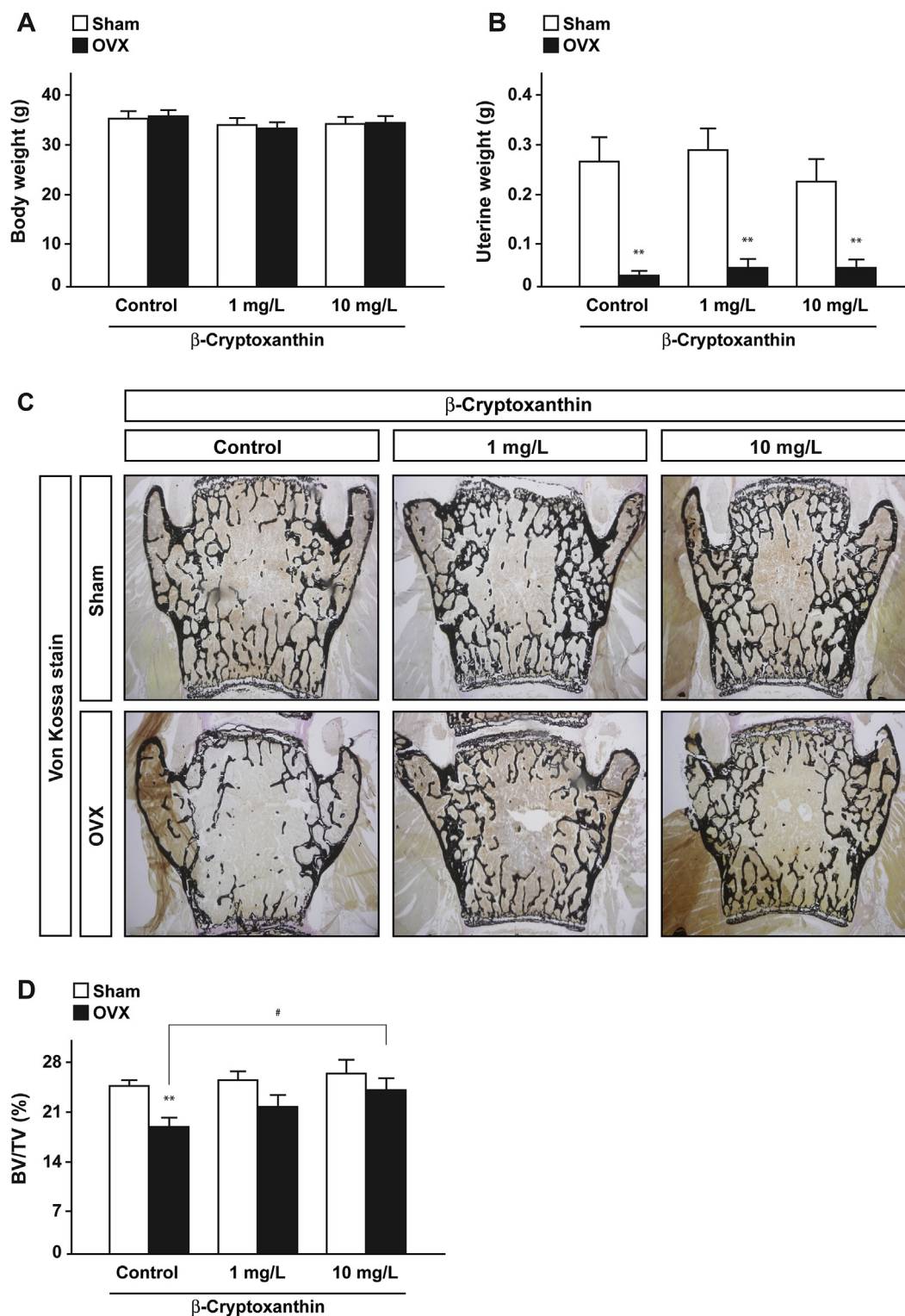


Fig. 1. Oral supplementation of β -cryptoxanthin inhibits OVX-induced bone loss. Eight-week-old female ddY mice were subjected to OVX, followed by daily oral supplementation of β -cryptoxanthin at 1 mg/L and 10 mg/L for 28 consecutive days (sham-control, $n = 13$; sham- β -cryptoxanthin 1 mg/L, $n = 12$; sham- β -cryptoxanthin 10 mg/L, $n = 11$; OVX-control, $n = 12$; OVX- β -cryptoxanthin 1 mg/L, $n = 12$; OVX- β -cryptoxanthin 10 mg/L, $n = 12$). (A) Body weight and (B) uterine weight. Typical pictures of Von Kossa staining are shown in the panel (C), while quantitative BV/TV data are shown in the panel (D). ** $P < 0.01$, significantly different from each control value obtained in sham-operated mice. # $P < 0.05$, significantly different from the value obtained in OVX-control mice.

performed on the differentiation and maturation of osteoclasts cultured with RANKL alone, in contrast to the *in vivo* situation in which other cells besides osteoclasts are present and a variety of cytokines other than RANKL are expressed in the bone. One

possibility is that β -cryptoxanthin inhibits osteoclastic differentiation and maturation by preferential interference with signaling processes mediated by RANKL in osteoclasts. The present finding that β -cryptoxanthin significantly inhibited NF- κ B-luc activity only

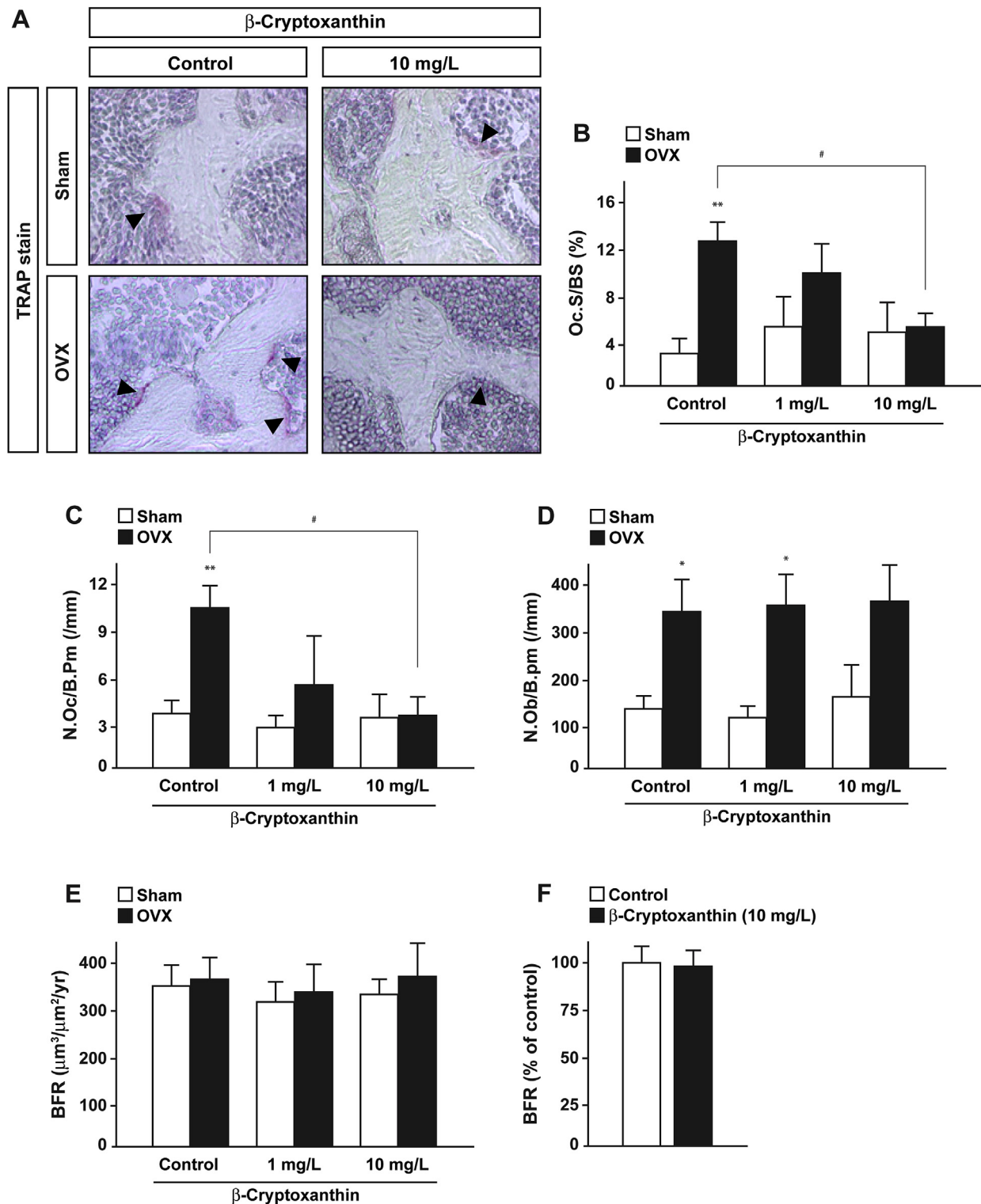


Fig. 2. Oral supplementation of β -cryptoxanthin inhibits OVX-induced osteoclastic activation. Eight-week-old female ddY mice were subjected to OVX, followed by oral supplementation of β -cryptoxanthin, and subsequent determination of different parameters for bone formation and resorption on 28 days after operation (sham-control, $n = 8$; sham- β -cryptoxanthin 1 mg/L, $n = 6$; sham- β -cryptoxanthin 10 mg/L, $n = 8$; OVX-control, $n = 8$; OVX- β -cryptoxanthin 1 mg/L, $n = 8$; OVX- β -cryptoxanthin 10 mg/L, $n = 8$). Typical pictures of TRAP staining are shown in the panel (A), while different quantitative analyses were done with determinations of (B) Oc.S/BS, (C) N.Oc/B.Pm, (D) N.Ob/B.Pm, and (E) BFR, respectively. * $P < 0.05$, ** $P < 0.01$, significantly different from each control value obtained in sham-operated mice. # $P < 0.05$, significantly different from the value obtained in OVX-control mice. (F) Eight-week-old female ddY mice were administrated β -cryptoxanthin and calcein, and subsequent determination of BFR (control, $n = 3$; β -cryptoxanthin 10 mg/L, $n = 3$).

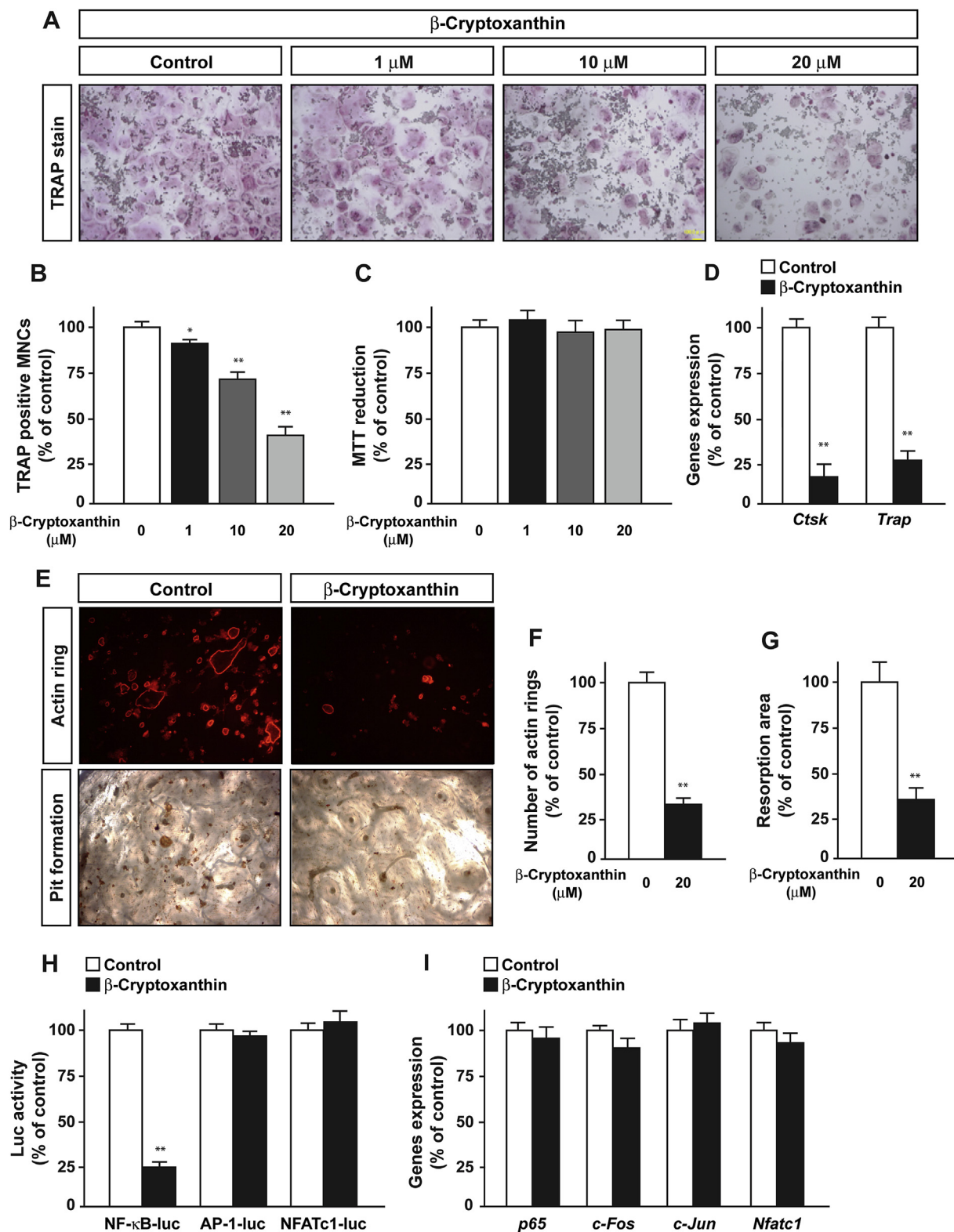


Fig. 3. β -cryptoxanthin inhibits osteoclastic differentiation and maturation without affecting cell survival. Pre-osteoclastic RAW264.7 cells were cultured with RANKL in either the presence or absence of β -cryptoxanthin at a concentration range from 1 μM to 20 μM , followed by determination of (A, B) the number of TRAP-positive MNCs, (C) MTT reduction and (D) genes expression. Quantitative data are shown in the panel (B), while typical pictures are shown in the panel (A). RAW264.7 cells were cultured with RANKL in either the presence or absence of β -cryptoxanthin, followed by (E, F) actin ring formation assay and (E, G) pit formation assay. Quantitative data are shown in the panel (F, G), while typical pictures are shown in the panel (E). (H) RAW264.7 cells were transiently transfected with the reporter plasmids, followed by culture in either the presence or absence of β -cryptoxanthin at 20 μM and subsequent further culture with RANKL for 24 h (I) RAW264.7 cells were cultured with RANKL in either the presence or absence of β -cryptoxanthin at 20 μM for 24 h, followed by determination of genes expression. * $P < 0.05$, ** $P < 0.01$, significantly different from each control value obtained in the absence of β -cryptoxanthin.

in the presence of RANKL in pre-osteoclastic RAW264.7 cells gives support to this idea.

Long-term intake of β -cryptoxanthin-rich foods gradually increases blood β -cryptoxanthin levels in humans and animals (4). On the basis of the present findings, we accordingly propose that appropriate consumption of β -cryptoxanthin-rich foods such as Satsuma mandarin (*Citrus unshiu* Marc.) would be beneficial for the maintenance of bone health and prophylaxis of menopausal osteoporosis, by a mechanism relevant to the inhibition of bone-resorbing osteoclast activation.

Conflict of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jphs.2015.08.003>.

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